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Original Article

Bacillus anthracis growth Inhibitory Properties of Australian *Terminalia* spp.: Putative Identification of low Polarity Volatile Components by GC-MS Headspace Analysis

Mitchell Henry Wright[1], Joseph Sirdaarta[1,2], Alan White[1], Anthony Carlson Greene[1], Ian Edwin Cock[1,2*]

[1]School of Natural Sciences, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, AUSTRALIA.
[2]Environmental Futures Research Institute, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, AUSTRALIA.

ABSTRACT

Introduction: Anthrax is a severe acute disease caused by *Bacillus anthracis* infections. If untreated, it often results in mortality. Many *Terminalia* spp. have documented therapeutic properties as general antiseptics, inhibiting the growth of a wide variety of bacterial species. This study examines the ability of selected Australian *Terminalia* spp. extracts to inhibit *B. anthracis* growth. **Methods:**Solvent extracts were prepared from *Terminalia carpentariae* and *Terminalia grandiflora* plant material and investigated by disc diffusion assay for the ability to inhibit the growth of an environmental strain of *B. anthracis*. Their MIC values were determined to quantify and compare their efficacies. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. The most potent extracts were analysed by GC-MS headspace analysis. **Results:** *T. carpentariae* and *T. grandiflora* leaf, fruit and nut solvent extractions displayed good growth inhibitory activity against *B. anthracis*. Methanolic *T. carpentariae* leaf and *T. grandiflora* nut extracts were particularly potent growth inhibitors, with MIC values of 74 and 155 µg/mL respectively. The *T. carpentariae* leaf ethyl acetate extract was also a good inhibitor of *B. anthracis* growth (MIC 340 µg/mL). All other extracts were substantially less potent growth inhibitors. Interestingly, the *T. carpentariae* leaf extracts with growth inhibitory activity were nontoxic in the *Artemia fransiscana* bioassay, with LC_{50} values >1000 µg/mL. In contrast, the LC_{50} value 740 µg/mL reported for the methanolic *T. grandiflora* nut extract indicates low-moderate toxicity. Non-biased GC-MS phytochemical analysis of the most active extracts (methanolic *T. carpentariae* leaf and *T. grandiflora* nut) putatively identified and highlighted several compounds that may contribute to the ability of these extracts to inhibit the growth of *B. anthracis*. **Conclusion:** The growth inhibitory activity of the methanolic *T. carpentariae* leaf and *T. grandiflora* nutextracts against *B. anthracis* indicates their potential for the treatment and prevention of anthrax. Furthermore, the lack toxicity of the *T. carpentariae* leaf and the low-moderate toxicity of the *T. grandiflora* nut extract, indicates that their use may extend to all forms of the disease (cutaneous, inhalation or gastrointestinal).

Key words: *Combretaceae*, *Terminalia carpentariae*, *Terminalia grandiflora*, Wild peach, Native almond, Anthrax, Metabolomic profiling.

Correspondence:
Dr. Ian Edwin Cock,
School of Natural Sciences,
Environmental Futures Research Institute ,
Nathan Campus, Griffith University,
170 Kessels Rd, Nathan,
Queensland 4111, AUSTRALIA.
Tel no.: +61 7 37357637; Fax no: +61 7 37355282.

Email: I.Cock@griffith.edu.au (I. E. Cock)
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INTRODUCTION

Bacillus anthracis is a gram-positive, endospore-forming bacterium and is the etiological agent of the disease anthrax. The disease has extensive implications in the livestock industry through the infection of grazing animals, however it is perhaps most commonly associated with its use in bioterrorism.[1] The most notable recent instance of weaponised anthrax occurred in 2001: *B. anthracis* spores were mailed to several locations in the U.S. and resulted in the infection of many people.[2] However, inadvertent mass infections can be traced as far back as ancient Egypt and it is theorised that the plagues described in ancient literature may have been anthrax mass infections.[3] Human anthrax is relatively rare compared to other vertebrates, and indeed outbreaks in both wildlife and livestock are a significant health and economic issue in many parts of the world.[4] Anthrax infection in humans occurs when *B. anthracis* endospores enter the body through inhalation, ingestion or through abrasions in the skin.[5,6] Once internalised, the body elicits an immune response, however the encapsulating endospore coating provides protection for the bacterium and can contribute to germination (via a process known as macrophage-enhanced germination).[7] The bacterium then resumes normal metabolic function and toxins are subsequently produced. Inhalation anthrax is the most dangerous of the three forms of the disease and infection via this pathway often results in death unless rapid treatment is administered.

Current strategies for the treatment of anthrax rely on the administration of both oral and intravenous antibiotics. Although vaccines have been available since the 19th century, they must be administered prior to infection and are generally ineffective in the treatment of anthrax once infection has initiated.[8] Whilst current antibiotic treatments are effective, due to the nature of antibiotics there is an inherent risk of *B. anthracis* conferring drug resistances and thus it is important to search for new antibiotics.[9] Antibiotic therapy development may occur via the design and synthesis of new chemical agents, and also through the investigation and discovery of natural resources for use as antimicrobial agents. Furthermore, the development of novel anti-*B. Anthracis* products that could disinfect contaminated sites without the use of harsh chemicals offers an effective, safe alternative of decreasing the spread of the disease.

Plants of the genus *Terminalia* have extensive therapeutic uses in multiple traditional healing systems, including uses for the prevention andtreatment of pathogenic diseases. Multiple studies have reported the antibacterial properties of *Terminalia* species used in traditional Indian medicine. Leaf and bark extracts of *Terminalia arjuna* have growth inhibitory activity against a wide panel of microbes.[10,11] *Terminalia chebula* also has a tradition of use in Ayurveda for the treatment of numerous diseases and conditions[12-14] and has also been reported to display potent

antibacterial activity against a microbial panel.[15] Similarly, *Terminalia alata*, *Terminalia bellirica* and *Terminalia catappa* have also been reported to have broad spectrum antibacterial activity.[11] Numerous African *Terminalia* species also have potent antibacterial activity. One study of the South East African species *Terminalia stenostachya* and *Terminalia spinosa* reported strong inhibitory activity against a broad spectrum of medicinally important bacteria including several *Mycobacterium* species, *Streptococcus faecalis*, *Staphylococcus aureus*, *Vibrio cholera*, *Bacillus anthracis*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Escherichia coli.*[16] The Southern African species *Terminalia sericea* and *Terminalia pruinoides* have similarly potent inhibitory activity against a broad panel of pathogenic[17] and food spoilage bacteria,[18] as well as against bacteria associated with autoimmune diseases.[19,20] *Terminalia brownii* also has a history of usage in traditional eastern and central African medicinal systems, including usage for the treatment of diverse medicinal conditions including diarrhoea and gonorrhoea.[21,22] Interestingly, a recent study also reported that the *T. brownie* was also a potent inhibitor of *B. anthracis* growth.[16] The Australian *Terminalia* species *Terminalia ferdinandiana* also has strong antibacterial activity against an extensive panel of bacteria.[23-26] Other *Terminalia* spp. which are endemic to the tropical northern regions of Australia also have a history of traditional therapeutic usage to treat microbial infections.[27] However, few studies have rigorously evaluated their therapeutic potential. This study screened two Australian *Terminalia* species (*T. carpentariae* and *T. grandiflora*) for the ability to inhibit *B. anthracis* growth.

MATERIALS AND METHODS

Plant source and extraction

The *Terminalia carpentariae* leaf and *Terminalia grandiflora* fruit and nut (seed) plant materials used in this study were a kind gift from David Boehme of Northern Territory Wild Harvest. Voucher samples of all plant specimens have been stored at the School of Natural Sciences, Griffith University, Brisbane (Australia). The plant materials were comprehensively desiccated in a Sunbeam food dehydrator and dried materials stored at -30ºC for later use. Prior to usage, the materials were thawed and ground into a coarse powder. Individual 1 g quantities of the materials were weighed into individual tubes and 50 mL of deionised water, methanol, hexane, chloroform or ethyl acetate were added. All solvents were obtained from Ajax Australia and were AR grade. The deionised water was sterilised prior to use. The ground plant materials were individually extracted in each solvent for 24 h at 4ºC through gentle shaking. The extracts were then filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant extract was weighed and redissolved in 10 mL deionised water (containing 1% DMSO).

Qualitative phytochemical studies

Phytochemical analysis of each extract for the presence of alkaloids, anthraquinones, cardiac glycosides, flavonoids, phenolic compounds, phytosterols, saponins, tannins andtriterpenoids was achieved as per previously described assays.[28-30]

Antibacterial screening

Environmental Bacillus anthracis strain

An environmental strain of *Bacillus anthracis* was isolated as previously described.[31] All growth studies were performed using a modified peptone/yeast extract (PYE) agar: 1 g/L peptone, 1.5 g/L yeast extract, 7.5 g/L NaCl, 1 g/L ammonium persulfate, 2.4 g/L HEPES buffer (pH 7.5) and 16 g/L bacteriological agar when required. Incubation was at 30ºC and the stock culture was subcultured and maintained in PYE media at 4ºC. The media nutrient components were supplied by Oxoid Ltd, Australia. The GenBank accession number for the 16S rRNA gene sequence for the isolate is KR003287.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[32,33] Briefly, 100 µL of established *Bacillus anthracis* culture was grown in 10 mL of fresh PYE liquid media until it reached a count of ~10^8 cells/mL. A 100 µL volume of bacterial suspension was spread onto PYE agar plates. The extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were subsequently impregnated with 10 µL of the test extract, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4ºC for 2 h before incubation at 30ºC for 24 h. The diameters of the zones of inhibition were measured in millimetres and all measurements were to rounded to the closest whole millimetre. Each assay was performed in triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (10 µg) and penicillin (2 µg) were obtained from Oxoid Ltd, Australia and served as positive controls for antibacterial activity. Filter discs impregnated with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of each extract was determined as previously described.[34] Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were impregnated with 10 µL of the test dilution, allowed to dry and placed onto inoculated plates. The assay was performed as described above and graphs of inhibition zones versus concentration were plotted for each extract. Linear regression was used to determine the MIC values.

Toxicity screening

Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 4 mg/mL solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was assessed using a modified *Artemia franciscana* nauplii lethality assay[35-37] Briefly, 400 µL of seawater containing ~43 (mean 43.2, n=155, SD 14.5) *A. franciscana* nauplii were added to wells of a 48 well plate and used for bioassay. A volume of 400 µL of each diluted plant extract or the reference toxin were transferred to the wells and incubated at 25 ± 1ºC under artificial light (1000 Lux). A negative control (400 µL seawater) was run in triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. Nauplii were considered dead if no movement of the appendages was observed within 10 sec. After 24 h all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was determined using probit analysis.

Non-targeted GC-MS head space analysis

Separation and quantification were performed using a Shimadzu GC-2010 plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass selective detector system as previously described.[23] The system was equipped with a Shimadzu auto-sampler AOC-5000 plus (USA) fitted with a solid phase micro-extraction fibre (SPME) handling system utilising a Supelco (USA) divinyl benzene/carbowax/polydimethylsiloxane (DVB/CAR/PDMS). Chromatographic separation was accomplished using a 5% phenyl, 95% dimethylpolysiloxane (30 m×0.25 mm id×0.25 um) capillary

column (Restek USA). Helium (99.999%) was employed as a carrier gas at a flow rate of 0.79 mL/min. The injector temperature was set at 230°C. Sampling utilised a SPME cycle which consisted of an agitation phase at 500 rpm for a period of 5 sec. The fibre was exposed to the sample for 10 min to allow for absorption and then desorbed in the injection port for 1 min at 250°C. The initial column temperature was held at 30°C for 2 min, increased to 140°C for 5 min, then increased to 270°C over a period of 3 mins and held at that temperature for the duration of the analysis. The GC-MS interface was maintained at 200°C with no signal acquired for a min after injection in split-less mode. The mass spectrometer was operated in the electron ionisation mode at 70 eV. The analytes were then recorded in total ion count (TIC) mode. The TIC was acquired after a min and fora duration of 45 mins utilising a mass range of 45-450 m/z.

Statistical analysis

Data is expressed as the mean ± SEM of at least three independent experiments.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of the various dried *Terminalia* materials with the solvents yielded dried plant extracts ranging from 16 mg (*T. grandiflora* nut ethyl acetate extract) to 348 mg (*T. carpentariae* leaf methanolic extract) (Table 1). The leaf extracts generally gave relatively high yields of dried extracted material compared to the fruit and nut extracts. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO) resulting in the extract concentrations shown in Table 1.

Qualitative phytochemical studies showed that methanol and water extracted the greatest amount and widest range of phytochemicals (Table 1). These solvents extracted high levels of water soluble phenolics, moderate to high levels of tannins, as well as low levels of flavonoids and anthraquinones for all *Terminalia* samples tested. Saponins were also generally present in the methanolic and aqueous extracts, although the levels of this class of compound were more variable. The ethyl acetate extracts generally extracted similar but lower phytochemical profiles as the methanolic and aqueous extracts. In contrast, the chloroform and hexane extracts were devoid of detectable levels of all classes of phytochemical screened for.

Antimicrobial activity

To determine the ability of the crude plant extracts to inhibit the growth of *B. anthracis*, aliquots (10 µL) of each extract were screened using a disc diffusion assay. The bacterial growth was inhibited by 7 of the 14 extracts screened (50%) (Figure 1). *T. carpentariae* methanolic leaf extract was the most potent inhibitor of *B. anthracis* growth (as judged by zone of inhibition), with inhibition zones of 13 ± 0.6 mm. This compares favourably with the penicillin and ampicillin controls, with zones of inhibition of 8.3 ± 0.6 and 10.0 ± 0.7 respectively. The *T. grandiflora* methanolic nut and *T. carpentariae* methanolic leaf extracts both displayed good inhibition of *B. anthracis* growth, with ≥ 10 mm zones of inhibition. In general, the methanolic extracts were more potent inhibitors of *B. anthracis* growth than were their counterparts.

The antimicrobial efficacy was further quantified by determining the MIC values (Table 2). Several of the extracts were effective at inhibiting microbial growth, with MIC values against *B. anthracis* substantially <1000 µg/mL (<10 µg impregnated in the disc). The methanolic *T. grandiflora* nut and *T. carpentariae* leaf extracts extracts were particularly potent, with MIC values of 155 and 74 µg/mL respectively (approximately 1.6 and 0.7 µg impregnated in the disc respectively). The *T. carpentariae* leaf ethyl acetate extract was also a potent inhibitor of *B. anthracis* growth (MIC value 340 µg/mL; 3.4 µg impregnated in the disc). The *T. grandiflora* fruit methanolic extract also had moderate growth inhibitory activity (MIC 3872 µg/mL). All other extracts were either unable to inhibit *B. anthracis* growth, or only displayed low inhibitory efficacy (MIC values >5000 µg/mL).

Quantification of toxicity

All extracts were initially screened at 2000 µg/mL in the assay (Figure 2). For comparison, the reference toxin potassium dichromate (1000 µg/mL) was also tested in the bioassay. Potassium dichromate was rapid in its onset of mortality, inducing nauplii death within the first 3 h of exposure and 100% mortality was evident following 4-5 h (results not shown). Most of the extracts displayed >75 % mortality at 24 h.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay. Table 2 shows the LC_{50} values of the extracts towards *A. franciscana*. No LC_{50} values are reported for the *T. grandiflora* nut chloroform, hexane and ethyl acetate extracts, nor for the *T. carpentariae* leaf hexane extract as <50 % mortality was seen for all concentrations tested. Significant toxicity was noted for the *T. grandiflora* nut, fruit and leaf methanolic extracts, with LC_{50} values substantially <1000 µg/mL. All other extracts were determined to be nontoxic, with LC_{50} values substantially greater than 1000 µg/mL following 24 h exposure. Extracts with an LC_{50} of greater than 1000 µg/mL towards *Artemia* nauplii have been defined as being nontoxic.[38]

Non-targeted GC-MS headspace analysis Australian *Terminalia* extracts

As the methanolic *T. carpentariae* leaf extract and the methanolic *T. grandiflora* nut extract had the most potent *B. anthracis* growth inhibitory efficacy (as determined by MIC; Table 2), they were deemed the most promising extracts for further phytochemical analysis. Optimised GC-MS parameters were developed and used to examine the phytochemical composition of these extracts. The resultant gas chromatograms for the methanolic *T. carpentariae* leaf extract and the methanolic *T. grandiflora* nut extract are presented in Figures 3 and Figure 4 respectively. Major peaks were evident in the methanolic *T. carpentariae* leaf extract at approximately 11.1, 12.9, 14.4, 17.0, 18.2 and 19.5 min (Figure 3). Several smaller peaks were also evident throughout all stages of the chromatograms. In total, 55 unique mass signals were noted for the methanolic *T. carpentariae* leaf extract (Table 3). Putative empirical formulas and identifications were achieved for 21 (38%) of these compounds by comparison with the database.

The methanolic *T. grandiflora* nut extract was also a potent inhibitor of *B. anthracis* growth (as determined by MIC; Table 2) and was therefore also analysed by headspace GC-MS with comparison to a GC-MS spectral database. The resultant gas chromatogramis presented in Figure 4. Several major peaks were present at times which also corresponded to peaks in the *T. carpentariae* leaf extract chromatogram (11.1, 12.9, 14.4 and 19.5 min). Several smaller peaks were also evident throughout all stages of the chromatograms. In total, 28 unique mass signals were noted for the methanolic *T. grandiflora* nutextract (Table 4). Putative empirical formulas and identifications were achieved for 11 (39%) of these compounds.

DISCUSSION

Many *Terminalia* spp. have a history of therapeutic usage to treat microbial infections and numerous recent investigations have reported on their

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *Terminalia* extracts. W = aqueous extract M = methanolic extract; C = chloroform extract; H = hexane extract; E = ethyl acetate extract

Species	Plant Part Used	Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/ml)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Polysteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones
T. grandiflora	nut	W	24	2.4	+++	++	-	-	++	-	-	-	-	+	++	+
T. grandiflora	nut	M	34	3.4	+++	++	-	-	+++	-	-	-	-	+	++	+
T. grandiflora	nut	C	126	12.6	-	-	-	-	-	-	-	-	-	-	-	-
T. grandiflora	nut	H	103	10.3	-	-	-	-	-	-	-	-	-	-	-	-
T. grandiflora	nut	E	16	1.6	+	+	-	-	-	-	-	-	-	+	+	+
T. grandiflora	fruit	W	80	8	+++	+++	-	-	++	+	-	-	+	+	+++	+
T. grandiflora	fruit	M	76	7.6	+++	+++	-	-	+++	+	-	-	+	+	+++	+
T. grandiflora	leaf	W	179	17.9	+++	+++	-	-	+	+	-	-	-	+	+++	+
T. grandiflora	leaf	M	293	29.3	+++	+++	-	+	++	+	-	-	-	+	+++	+
T. carpentariae	leaf	W	180	18	+++	+++	-	-	-	-	-	-	+	+	+++	+
T. carpentariae	leaf	M	348	34.8	+++	+++	-	+	+	-	-	-	-	+	+++	+
T. carpentariae	leaf	C	200	20	-	-	-	-	-	-	-	-	-	-	-	-
T. carpentariae	leaf	H	182	18.2	-	-	-	-	-	-	-	-	-	-	-	-
T. carpentariae	leaf	E	42	4.2	++	+	-	-	-	-	-	-	-	+	-	+

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

Table 2: Minimum inhibitory concentration (µg/mL) of the plant extracts and LC_{50} values (µg/mL) in the *Artemia* nauplii bioassay. W = aqueous extract M = methanolic extract; C = chloroform extract; H = hexane extract; E = ethyl acetate extract

Species	Part	Extract	MIC (µg/mL)	LC50 (µg/mL)
T. grandiflora	nut	W	-	1488
T. grandiflora	nut	M	155	740
T. grandiflora	nut	C	-	-
T. grandiflora	nut	H	-	-
T. grandiflora	nut	E	-	-
T. grandiflora	fruit	W	5380	3124
T. grandiflora	fruit	M	3872	370
T. grandiflora	leaf	W	-	1643
T. grandiflora	leaf	M	-	734
T. carpentariae	leaf	W	>10,000	1336
T. carpentariae	leaf	M	74	1160
T. carpentariae	leaf	C	-	15358
T. carpentariae	leaf	H	>10,000	-
T. carpentariae	leaf	E	340	1189
Potassium Dichromate	-	-		82

Numbers indicate the mean MIC and LC_{50} values of triplicate determinations. - indicates no bacterial growth inhibition was evident, or that an LC_{50} value could not be obtained as the mortality did not reach 50 % for any dose tested.

Table 3: GC-MS headspace analysis of the *T. carpentariae* leaf methanolic extract, elucidation of empirical formulas and putative identification (where possible) of each compound

Molecular Mass	Molecular Formula	Retention Time (min)	Area%	Height%	Putative Identification
151	$C_8H_9NO_2$	11.094	24.64	19.58	Methyl N-hydroxybenzene carboximidoate
		11.972	1.73	0.52	
		12.933	6.08	5.08	
128	$C_8H_{16}O$	13.679	0.96	0.68	1-Octen-3-ol
126	$C_8H_{14}O$	13.94	1.2	1.01	5-Hepten-2-one, 6-methyl-
118	$C_6H_{14}O_2$	14.51	1.3	1.18	2-tert-Butoxyethanol
130	$C_8H_{18}O$	15.375	0.77	0.78	2-Ethyl-1-hexanol
146	$C_6H_{10}O_4$	15.512	1.45	1.03	Dimethyl succinate
138	$C_9H_{14}O$	16.444	0.2	0.25	Isophorone
156	$C_{10}H_{20}O$	16.665	0.38	0.19	α-Citronellol
		16.881	0.76	0.81	
		17.331	0.42	0.21	
		17.445	0.13	0.06	
142	$C_9H_{18}O$	17.877	0.91	1.02	Nonanal
		18.065	1.48	1.97	
		18.189	4.22	5.2	
152	$C_9H_{12}O_2$	19.18	0.28	0.33	4-Oxoisophorone
		19.281	0.49	0.72	
		19.45	0.35	0.49	
150	$C_9H_{10}O_2$	20.061	0.47	0.61	Ethyl benzoate
150	$C_9H_{10}O_2$	20.267	0.57	0.63	Methyl benzeneacetate
154	$C_{10}H_{18}O$	20.731	0.14	0.21	α.-Terpineol
150	$C_{10}H_{14}O$	21.032	0.34	0.43	2-Isopropylidene-3-methylhexa-3,5-dienal
184	$C_{12}H_{24}O$	21.104	0.16	0.22	Lauraldehyde
		21.186	0.29	0.4	
		21.254	0.75	0.81	
134	$C_9H_{10}O$	21.485	1.26	1.36	2,4-Dimethylbenzaldehyde
		21.842	0.29	0.27	
		22.486	2.64	2.11	
		22.799	0.5	0.48	
		22.944	0.13	0.21	
		23.419	0.12	0.23	
		23.579	0.09	0.15	
		24.56	0.21	0.22	
		25.398	1.21	1.25	
		25.704	0.31	0.37	
216	$C_{12}H_{24}O_3$	26.434	0.51	0.5	1,3-Pentanediol, 2,2,4-trimethyl-, 1-isobutyrate
		27.016	0.31	0.31	
		27.523	0.29	0.29	
		27.601	0.42	0.41	
336	$C_{20}H_{26}F_2O_2$	31.363	2.81	3.1	2-Tridecynyl 2,6-difluorobenzoate
206	$C_{14}H_{22}O$	31.643	1.75	2	2,4-Di-tert-butylphenol
194	$C_{11}H_{14}O_3$	32.057	3.11	3.17	Benzoic acid, 4-ethoxy-, ethyl ester
		32.334	0.26	0.19	
		32.73	0.32	0.22	
		32.86	0.21	0.25	
		33.111	0.28	0.27	
286	$C_{16}H_{30}O_4$	33.826	0.85	1.2	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate
		36.207	0.07	0.12	
		38.297	0.15	0.22	
		39.563	0.12	0.23	
		41.192	0.12	0.24	
		42.634	0.36	0.22	
		44.176	0.12	0.21	

The % area and % height is expressed as a % of the total area under all chromatographic peaks or % of the total height of all peaks respectively.

Table 4: GC-MS headspace analysis of the *T. grandiflora* nut methanolic extract, elucidation of empirical formulas and putative identification (where possible) of each compound

Molecular Mass	Molecular Formula	Retention Time (min)	Area%	Height%	Putative Identification
151	$C_8 H_9 NO_2$	11.073	24.94	14.96	Methyl N-hydroxybenzene carboximidoate
		12.927	6.35	5.28	
118	$C_6 H_{14} O_2$	14.502	1.53	1.25	Ethanol, 2-(1,1-dimethylethoxy)-
130	$C_8 H_{18} O$	15.371	0.48	0.54	2-ethyl-1-Hexanol
142	$C_9 H_{18} O$	17.866	0.64	0.84	Nonanal
		18.053	1.23	1.56	
		18.178	4.34	5.47	
150	$C_9 H_{10} O_2$	19.27	0.62	0.89	Ethyl benzoate
		20.051	0.26	0.37	
		21.085	0.25	0.18	
		21.243	0.35	0.42	
		22.48	1.78	1.49	
		22.79	0.16	0.29	
		23.407	0.15	0.27	
		25.38	0.82	0.84	
216	$C_{12} H_{24} O_3$	26.414	0.49	0.52	1,3-Pentanediol, 2,2,4-trimethyl-, 1-isobutyrate
206	$C_{14} H_{22} O$	31.629	1.44	1.6	2,4-Di-tert-butylphenol
194	$C_{11} H_{14} O_3$	32.045	1.49	1.55	Benzoic acid, 4-ethoxy-, ethyl ester
204	$C_{15} H_{24}$	33.018	0.78	1.06	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene- (caryophyllene)
286	$C_{16} H_{30} O_4$	33.812	1.04	1.47	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate
334	$C_{20} H_{30} O_4$	37.642	0.2	0.4	Butyl octyl phthalate
		38.285	0.16	0.27	
		39.552	0.1	0.26	
		41.183	0.12	0.22	
		42.399	0.67	0.59	
		42.616	0.27	0.29	
		43.8	0.15	0.12	
		44.162	0.34	0.27	

The % area and % height is expressed as a % of the total area under all chromatographic peaks or % of the total height of all peaks respectively.

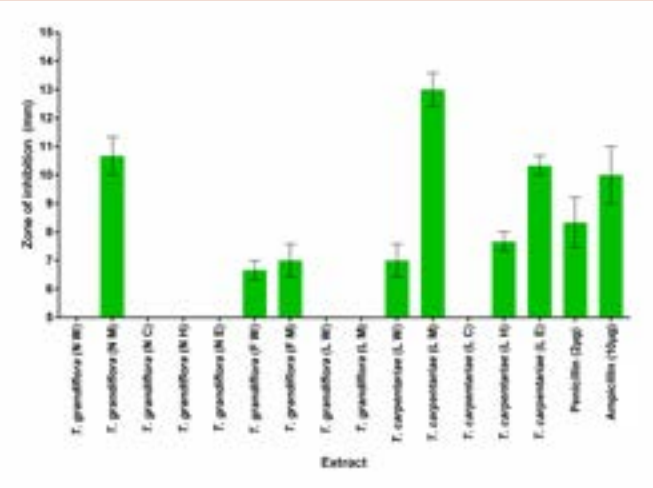

Figure 1: Growth inhibitory activity of *Terminalia* spp. extracts against the *B. anthracis* environmental isolate measured as zones of inhibition (mm). N=nut; F=fruit; L=leaf; W=aqueous extract M=methanolic extract; C=chloroform extract; H=hexane extract; E=ethyl acetate extract. Results are expressed as mean zones of inhibition ± SEM.

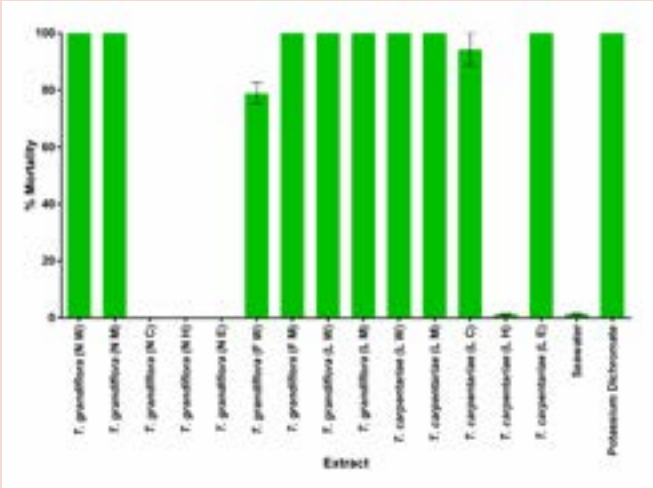

Figure 2: The lethality of *Terminalia* spp. extracts (2000 µg/mL) and the potassium dichromate (1000 µg/mL) and seawater controls towards *A. franciscana* nauplii after 24 h exposure. N=nut; F=fruit; L=leaf; W=aqueous extract M=methanolic extract; C=chloroform extract; H=hexane extract; E=ethyl acetate extract. Results are expressed as mean % mortality ± SEM.

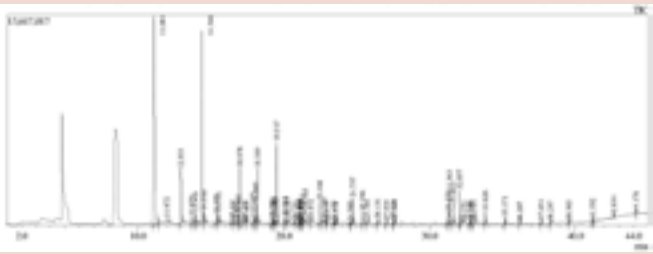

Figure 3: GC headspace chromatograms of 0.5 µL injection of *T. carpentariae* leaf methanolic extract. The extract was dried and resuspended in methanol for analysis.

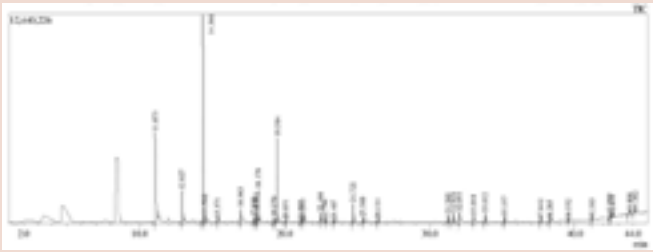

Figure 4: GC headspace chromatograms of 0.5 µL injection of *T. grandiflora* nut methanolic extract. The extract was dried and resuspended in methanol for analysis.

antibacterial properties.[39] Of the Australian species, *T. ferdinandiana* has been the most extensively studied. Several studies have reported it to be a potent antibacterial agent, with growth inhibitory activity reported against a broad panel of bacterial pathogens,[26] as well as against some bacterial triggers of rheumatoid arthritis[23,25] and multiple sclerosis.[24,25] Furthermore, *T. ferdinandiana* has also recently been reported to inhibit the proliferation of the gastrointestinal protozoan parasite *Giardia duodenalis*[40] indicating its therapeutic potential against both prokaryotic and eukaryotic pathogens. Interestingly, whilst inhibition of *B. anthracis* growth was not evaluated in any of the previous studies, one recent study reported potent growth inhibition of the related bacterial species *B. cereus*, with MIC values as low as approximately 100 µg/mL.[26] *B. cereus* is very closely related to *B. anthracis* with >99% 16S rRNA gene sequence homology[41] and some bacterial taxonomonists believe that they should be classified as a single species under current standards (>97% 16S rRNA sequence homology). In contrast, other native Australian *Terminalia* spp. are less well studied.

The methanolic *T. carpentariae* leaf and *T. grandiflora* nutextracts displayed the most potent *B. anthracis* growth inhibitory activity (MIC values of 74 and 155 µg/mL respectively) and were therefore analysed by qualitative GC-MS. A number of interesting compounds were identified in each of these extracts. Analysis of the methanolic *T. carpentariae* leaf extract putatively identified methyl N-hydroxybenzenecarboximidoate (Figure 5a), 1-octen-3-ol (Figure 5b), 5-hepten-2-one, 6-methyl- (Figure 5c), 2-tert-butoxyethanol (Figure 5d), 2-ethyl-1-hexanol (Figure 5e), dimethyl succinate (Figure 5f), isophorone (Figure 5g), α-citronellol (Figure 5h), nonanal (Figure 5i), 4-oxoisophorone (Figure 5j), ethyl benzoate (Figure 5k), methyl benzeneacetate (Figure 5l), α-terpineol (Figure 5m), 2-isopropylidene-3-methylhexa-3,5-dienal (Figure 5n), lauraldehyde (Figure 5o), 2,4-dimethyl-benzaldehyde (Figure 5p), 1,3-pentanediol, 2,2,4-trimethyl-, 1-isobutyrate (Figure 5q), 2,4-di-tert-butylphenol (Figure 5r), ethyl para-ethoxybenzoate (Figure 5s) and2,2,4-trimethyl-1,3-pentanediol diisobutyrate (Figure 5t). The presence of the monoterpenoids α-citronellol and α-terpineol are particular interesting as many monoterpenoids have potent broad spectrum antibacterial activity[42] and therefore may contribute to the *B. anthracis* growth inhibition reported in our study. Interestingly, several monoterpenes have also been reported to suppress NF-κB signalling (the major regulator of inflammatory diseases).[43,44] Thus, the terpene components may have a pleuripotent mechanism in blocking anthrax, by inhibiting the growth of the causative bacterium, as well as relieving the downstream inflammatory symptoms evident with the most common (cutaneous) form of the disease.

Many of the same compounds detected in the methanolic *T. carpentariae* leaf extract were also putatively identified in the methanolic *T. grandi-*

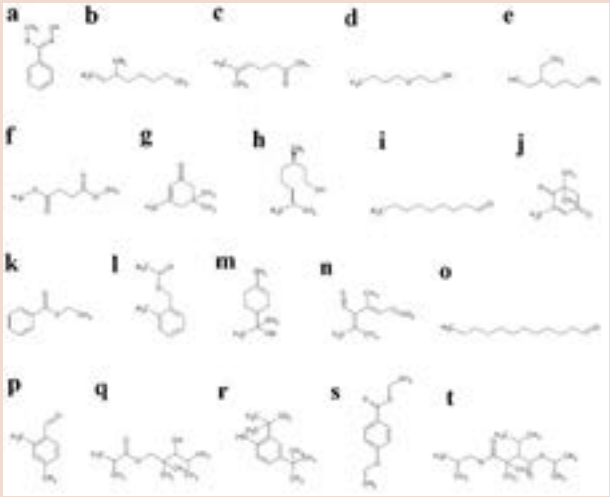

Figure 5: Methanolic *T. carpentariae* leaf components: (a) methyl N-hydroxybenzenecarboximidoate, (b) 1-octen-3-ol, (c) 5-hepten-2-one, 6-methyl-, (d) 2-tert-butoxyethanol, (e)2-ethyl-1-hexanol, (f) dimethyl succinate, (g)isophorone, (h) α-citronellol, (i) nonanal, (j) 4-oxoisophorone, (k) ethyl benzoate, (l) methyl benzeneacetate, (m) α-terpineol, (n) 2-isopropylidene-3-methylhexa-3,5-dienal, (o) lauraldehyde, (p) 2,4-dimethyl-benzaldehyde, (q) 1,3-pentanediol, 2,2,4-trimethyl-, 1-isobutyrate, (r) 2,4-di-tert-butylphenol, (s) ethyl para-ethoxybenzoate, (t) 2,2,4-trimethyl-1,3-pentanediol diisobutyrate.

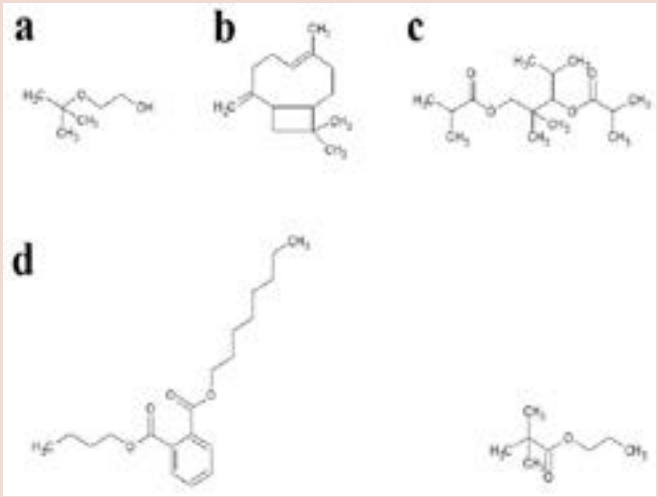

Figure 6: Methanolic *T. grandiflora* nut components not also detected in the methanolic *T. carpentariae* leaf extract: (a) 2-(1,1-dimethylethoxy)-ethanol, (b) caryophyllene, (c) 2,2,4-trimethyl-1,3-pentanediol diisobutyrate, (d) butyl octyl phthalate.

flora nut extract. In particular, methyl N-hydroxybenzenecarboximidoate, 2-(1,1-dimethylethoxy)-ethanol, 2-ethyl-1-hexanol, nonanal, ethyl benzoate, 1,3-pentanediol, 2,2,4-trimethyl-,1-isobutyrate, 2,4-di-tert-butylphenol and benzoic acid, 4-ethoxy-ethyl ester were also present in the methanolic *T. grandiflora* nut extract. GC-MS analysis also putatively identified 2-(1,1-dimethylethoxy)-ethanol (Figure 6a), caryophyllene (Figure 6b), 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (Figure 6c) and butyl octyl phthalate (Figure 6d) in the methanolic *T. grandiflora* nut extract. Previous studies have reported bacterial growth inhibitory activities for the sesquiterpenoid caryophyllene.[42] It is likely that caryophyllene therefore contributes (at least in part) to the growth inhibitory activity of this extract.

It is likely that other phytochemical classes also contribute to the growth inhibitory properties of these extracts. Our qualitative phytochemical screening studies indicate that polyphenolics, flavonoids, saponins, and tannins were present in the methanolic *T. carpentariae* leaf and *T. grandiflora* nut extracts. However, no compounds of these classes were identified by GC-MS headspace analysis. As GC-MS techniques generally only detect lower polarity compounds, many mid to higher polarity bioactive compounds may have been missed. Recent studies have reported the LC-MS profiles of extracts prepared from other Australian *Terminalia* spp.[23-25,40] Several features were common to all of these studies. In particular, all of these studies reported on the diversity of tannins in the *Terminalia* extracts. This is noteworthy as tannins have potent growth inhibitory activity against a broad spectrum of bacterial species.[39] Recent studies have also highlighted the stilbene components in extracts prepared from different Australian *Terminalia* spp.[24,25] Resveratrol and the glycosylated resveratrol derivative piceid, and several combretastatins were putatively identified in those studies. Stilbenes have attracted much recent interest due to their reported potent ability to of some compounds to block cancer cell progression and induce apoptosis by binding intracellular tubulin, thereby disrupting microtubule formation.[45] Further studies utilising LC-MS are required to identify the mid to higher polarity compounds in the methanolic *T. carpentariae* leaf and *T. grandiflora* nut extract for a more complete understanding of the complete plant metabolome.

Of note, the methanolic *T. carpentariae* leaf extract was determined to be nontoxic towards *Artemia franciscana* nauplii, with LC_{50} values >1000 µg/mL. Extracts with LC_{50} values >1000 µg/mL towards *Artemia* nauplii are defined as being nontoxic.[38] This indicates that this extract may be safe for use for all forms of the disease (cutaneous, inhalation or gastrointestinal). In contrast, the methanolic *T. grandiflora* nut extract (which also was a potent inhibitor of *B. anthracis* growth) displayed toxicity towards *Artemia* nauplii, with an LC_{50} value of 740 µg/mL. This represents low to moderate toxicity and indicates that this using human cell lines are required to further evaluate the safety of these extracts. Furthermore, whilst the results of our study are promising, it must be noted that the growth inhibitory studies screened against vegetative cells. As *Bacillus* spp. are spore formers, further studies are required to determine whether extracts with *B. anthracis* growth inhibitory activity also affect bacterial growth from the spores.

CONCLUSION

The *B. anthracis* growth inhibitory activity and low toxicity of the *T. carpentariae* and *T. grandiflora* extracts demonstrate their potential in the prevention and treatment of anthrax. Methanolic *T. carpentariae* leaf and *T. grandiflora* nut extracts were particularly potent growth inhibitors. Further investigations aimed at the purification of the bioactive components are needed to assess the mechanisms of action of these agents.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS USED

DMSO: Dimethyl sulfoxide; **LC_{50}:** The concentration required to achieve 50 % mortality; **MIC:** Minimum inhibitory concentration; **PYE:** Peptone yeast extract.

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PICTORIAL ABSTRACT

SUMMARY

- *T. carpentariae* and *T. grandiflora* extracts inhibited *Bacillus anthracis* growth *in vitro*.
- The methanolic *T. carpentariae* leaf and *T. grandiflora* nut extracts were particularly potent growth inhibitors with MIC's of 74 and 155 µg/mL respectively.
- Generally, the inhibitory *Terminalia* extracts were either non-toxic or low toxicity in the *Artemia* nauplii assay.
- GC-MS headspace profiling of the inhibitory extracts revealed distinct phytochemical profiles for the *T. carpentariae* and *T. grandiflora* extracts.
- Phytochemical profiling highlighted several nonpolar compounds as potentially contributing to the *B. anthracis* growth inhibitory activity.

ABOUT AUTHORS

Dr Mitchell Henry Wright: Received his PhD in 2014, for his work investigating the manganese reduction and oxidation characteristics of environmental bacteria. He is currently a postdoctoral researcher at Griffith University, Australia, where he is working on several projects both in the areas of geomicrobiology and pharmocognosy. His present research interests are the use of biogenic manganese oxides in the bioremediation of metal-contaminated sites as well as the use of Australian native plants in the treatment and prevention of various pathogenic bacteria.

Dr Anthony Greene: Is a senior lecturer and researcher at Griffith University, Brisbane Australia. He obtained his PhD in Microbiology from the University of New South Wales and focuses on extreme environments, bioremediation and Geomicrobiology. His specific interests include the microbial ecology of thermophilic, saline and alkaliphilic environments and the mechanisms and industrial potential of extremophilic bacteria contained therein.

Dr Ian Cock: Leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin including *Aloe vera*, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens, Pittosporum phyllireaoides, Terminalia ferdinandiana* (Kakadu plum), Australian Acacias, Syzygiums, Petalostigmas and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in nearly 200 scientific publications in a variety of peer reviewed journals.